Food and Chemical Toxicology 68 (2014) 142–153

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Reproductive toxicity in rats with crystal nephropathy following high doses of oral melamine or cyanuric acid

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ARTICLE INFO

Article history:
Received 24 January 2014
Accepted 23 February 2014
Available online 28 February 2014

Keywords:
Melamine
Cyanuric acid
Reproductive toxicity
Rats

ABSTRACT

The industrial chemical melamine was used in 2007 and 2008 to raise the apparent protein content in pet feed and watered down milk, respectively. Because humans may be exposed to melamine via several different routes into the human diet as well as deliberate contamination, this study was designed to characterize the effect of high dose melamine or cyanuric acid oral exposure on the pregnant animal and developing fetus, including placental transfer. Clear rectangular crystals formed following a single triazine exposure which is a different morphology from the golden spherulites caused by combined exposure or the calculi formed when melamine combines with endogenous uric acid. Crystal nephropathy, regardless of cause, induces renal failure which in turn has reproductive sequelae. Specifically, melamine alone-treated dams had increased numbers of early and late fetal deaths compared to controls or cyanuric acid-treated dams. As melamine was found in the amniotic fluid, this study confirms transfer of melamine from mammalian mother to fetus and our study provides evidence that cyanuric acid also appears in the amniotic fluid if mothers are exposed to high doses.

1. Introduction

Melamine is an industrial chemical used to manufacture plastics, laminates, resins, and fertilizers, but is also commonly found in dishware and kitchenware. Because it has high nitrogen content, melamine was fraudulently added to edible foodstuffs ostensibly to raise apparent protein levels during routine testing. During 2007, in North America and South Africa, wheat flour and rice protein concentrate destined for pet food were adulterated with recycled melamine that contained related analogs (such as ammeline, ammalide, and cyanuric acid). This adulteration caused hundreds of pet deaths due to renal failure. Microscopic melamine–cyanurate crystals obstructed renal tubules within the kidney, causing kidney damage by a mechanism similar to that of uric acid nephropathy (Reimschuessel et al., 2008). Altogether, thousands of pets were affected to some degree along melamine's toxicity spectrum from renal impairment to failure and death (Puschner and Reimschuessel, 2011). During 2008 in China, a more refined melamine was added to watered down milk, which in turn, was used to manufacture powdered infant formula. The melamine combined with endogenous uric acid to form calculi in the kidney, ureter, or bladder, and an estimated 294,000 infants were affected in the first months of the crisis (WHO, 2008).

In addition to infant formula and milk, other dairy products, beverages, eggs, and candies were contaminated with melamine (Ingelfinger, 2008; Xia et al., 2009). Further, additional exposure to leaching melamine may occur if chlorinated disinfectants are used when cleaning melamine dishware (WHO, 2008). Because of these many potential additional sources of melamine in the diet, there is concern that pregnant women could be exposed to melamine contaminated foods. The fate of melamine, and its related analogue cyanuric acid, in pregnant females and developing fetuses has not been fully characterized.

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http://dx.doi.org/10.1016/j.fct.2014.02.029
0278-6915/Published by Elsevier Ltd.

Previous studies in pregnant rats primarily focus on single doses to determine pharmacokinetic parameters of transfer or focus on melamine only (Chu et al., 2010; Jingbin et al., 2010; Chan et al., 2011; Kim et al., 2011; Chu et al., 2013). We designed our study to investigate the effects of repeated high doses of either melamine or cyanuric acid on pregnant rats and their developing fetuses. The study detailed herein reports the concentrations of melamine and cyanuric acid in the amniotic fluid and serum from these maternal rats and the effects of these triazines on fetal development and crystal formation in the maternal kidney.

2. Material and methods

2.1. Experimental animals and animal husbandry

Caesarean-derived CD IGS VAF/+ rats ($n = 35$ pregnant and $n = 33$ nulliparous non-pregnant rats) 8–10 weeks of age were purchased from Charles River (Frederick, MD, USA). The animals were acclimated for approximately 3 days at which point they were uniquely identified by ear tags. The experimental animals were individually housed in microisolator cages in an air-conditioned room maintained at 18–26 °C, and 40–70% relative humidity. A 12-h light and dark cycle was automatically maintained. Fluid and feed consumption was measured every 3 and 7 days, respectively. Feed (Harlan Tekland Global 2018CM rodent diet) and water (Hydro-Pico Systems, Inc., Research Triangle Park, NC, USA) were provided *ad libitum* throughout the study. The diet was confirmed negative for melamine, cyanuric acid, ammeline, and ammelide by a contract laboratory (Eurofins, New Orleans, LA, USA). Animal use and procedures were reviewed and approved by the USFDA/CFSAN Institutional Animal Care and Use Committee.

2.2. Test material

Dosing solutions (10% w/v) of melamine (MEL; 99% pure; Sigma–Aldrich, St. Louis, MO, USA) and cyanuric acid (CYA; 98% pure; Sigma–Aldrich, St. Louis, MO, USA) were prepared in 1% carboxymethylcellulose (CMC-Na; Sigma–Aldrich, St. Louis, MO, USA). The concentration of the dosing solutions was confirmed by a contract laboratory (Eurofins, New Orleans, LA, USA) using LC–MS/MS.

2.3. Experimental design

Time pregnant and non-pregnant animals, beginning on gestation day 10 (GD10), were randomly assigned to one of the following 6 dose groups by weight using a stratified random procedure: (1) non-pregnant rats administered 1% CMC-Na vehicle (control; $n = 11$), (2) non-pregnant rats ($n = 11$) administered 1000 mg/kg BW/day CYA, (3) non-pregnant rats ($n = 11$) administered 1000 mg/kg BW/day MEL, (4) time-pregnant rats administered 1% CMC-Na vehicle (control; $n = 11$), (5) time-pregnant rats ($n = 11$) administered 1000 mg/kg BW/day CYA, and (6) time-pregnant rats ($n = 13$) administered 1000 mg/kg BW/day MEL. Animals received the test article by gavage for 10 straight days in the morning at a volume of 1 mL/100 g body weight. The 1000 mg/kg BW/day dose was chosen because the purpose of this study was to determine whether a high dose of triazine (either MEL of CYA) could induce crystal formation and to determine if crystal formation was affected by pregnancy.

2.4. Clinical observations

Clinical observations were made at least twice on the first day of dosing and at least once daily thereafter throughout the gestational period. Observations included, but were not limited to, changes in skin, fur, eyes, and mucous membranes. Respiratory, circulatory, autonomic, and central nervous systems were observed, in addition to somatomotor activity and behavior patterns. Attention was directed toward observations of tremors, convulsions, salivation, diarrhea, lethargy, sleep, and coma. Animals taken off study early were considered equivocal to animals that died.

2.5. Tissue collection

2.5.1. Non-pregnant animals

Non-pregnant rats were euthanized by carbon dioxide asphyxiation after 10 consecutive days of exposure to the control or test material. Rats were bled from the inferior vena cava, and the samples were allowed to clot for 15 min, centrifuged for 15 min, and stored at −80 °C until analyses. The kidneys were weighed and either preserved in formalin or frozen for wet-mount analysis. Finally, remaining maternal organs were removed and weighed.

2.5.2. Pregnant animals

Pregnant females were euthanized by carbon dioxide asphyxiation on GD20. A mid ventral incision was made in the abdomen to expose the abdominal and pelvic viscera. The number of corpora lutea, the number of implantation sites, and the number and position of resorption sites and fetuses (dead or alive) were noted. The gravid uterus was removed *in toto* and weighed. Each viable fetus was removed from the uterus and examined individually and records were kept as to its uterine position, sex, weight, and crown-rump length, as well as any externally visible abnormalities. Runts, defined as an animal whose body weight is <70% of the average weight of the control litter averages by gender, were identified and their position noted. The uteri of animals that did not appear pregnant were stained with ammonium sulfide to enhance the observation of implantation sites. Early death was defined as a deciduoma, an implantation site with no fetal development. Late death was defined as fetuses that had shape or form, which indicates the implantation was successful and the fetus died later in gestation. A 20 gauge needle was inserted into the amniotic sac and amniotic fluid was aspirated. Each aspiration was observed for blood contamination before it was pooled by dam. Approximately 1.5 mL of amniotic fluid was collected per dam. Finally, remaining maternal organs were removed and weighed.

2.6. Wet-mount analysis

One half of the right kidney and the full left kidney were frozen until analysis. For wet-mount analysis, a thin slice of renal tissue containing portions of both medulla and cortex was weighed and then compressed between two microscope slides with a coverslip inserted between the tissue and one of the slides. Fresh slides were observed on a light microscope. The crystals observed were ranked on a subjective scale from 0 to 5 as follows: (0) none seen; (1) extremely few (1 or 2 in an entire section); (2) few with scattered distribution; (3) moderate numbers seen throughout section; (4) large numbers seen immediately; and (5) extensive numbers obliterating the regular tissue architecture. Kidneys from fetuses were not examined by wet-mount as results from a preliminary study (data not shown) indicate renal crystals are not formed in neonates or in fetuses from mothers exposed at these dose levels.

2.7. Formalin, ethanol, and freezing effects on crystals

Additional slices of kidney from rats exposed to MEL only and to CYA only with a crystal rank of 4 or 5 were prepared for wet-mount as described above. Slides were flooded with 10% neutral buffered formalin and photomicrographs were taken at 0, 1, 2, 3, and 24 h for the MEL kidney and 0, 10, 20, 30 min, and 1 h for the CYA kidney.

A similar experiment using ethanol instead of formalin was performed, and sections were evaluated daily for up to 6 days. Frozen sections were cut at 6 μm and either used unstained or were stained with hematoxylin and eosin.

2.8. Histopathology

One half of the right kidney and the bladder from each dam were preserved in 10% neutral buffered formalin and processed for routine embedding in paraffin. Sections were stained with hematoxylin and eosin.

2.9. Raman spectroscopy

Archived kidney samples were kept at −80 °C until analysis. Multiple thin sections were cut using a razor blade onto quartz slides with a thickness of 25 × 76 × 1 mm (SPI, West Chester, PA, USA). Prior to analysis with the Raman spectroscopy microscope, samples were confirmed to have crystals using light microscopy. Standards (ACROS Organics, NJ, USA) of melamine powder and cyanuric acid powder were run the same day by placing the powders on a quartz slide.

Samples were analyzed using a Raman spectroscopy microscope including an optical microscope (Olympus BX41) and a spectrograph (XPLORA, Horiba Jobin Yvon, Edison, NJ, USA). All sample analysis was completed using a 785 nm laser (20–25 mW laser power) with a 100 × objective for 20 s with 3 iterations. A 30 s photobleach was used to reduce fluorescence caused by tissue samples and was also used on standards to maintain consistency. A grating of 1200 lines/mm was used with a 500 μm hole and 200 μm slit. All spectra were collected using LabSpec 5 (HORIBA Jobin Yvon SAS, France).

A representative spectrum for each sample/standard was chosen. Raw spectra were automatically baseline corrected using OMNIC™ 8 software for dispersive Raman (Thermo Electron, Madison, WI, USA). In order to account for natural variations in signal intensity due to crystal formation and orientation in the tissue, each spectrum was normalized to highest intensity peak for ease of visual comparison and spectral library analysis. Data were plotted in GraphPad Prism (v. 5.02, La Jolla, CA, USA) with the individual spectra offset for visual clarity.